Select Bio Consult, LLC

Research Report: 1001F (Final)

Health, Safety, and Environmental Properties of the Green Algae Genus *Parachlorella* – (Final)

April 5, 2019

Prepared for:

David S. Hanselman, PhD

Senior Director of Regulatory Affairs and Quality

Synthetic Genomics, Inc.

11149 North Torrey Pines Road

La Jolla, CA 92037

Author:

Stephen Buxser, PhD

Scientific Director, Select Bio Consult, LLC

Phone: 269-216-7115

Email: sbuxser@selectbioconsult.com

Website: www.Selectbioconsult.com

Table of Contents

Executive Summary

Goals and Objectives

Synthetic Genomics, Inc. (SGI) is developing renewable biofuel using microalgae. As part of SGI's safety, health, and environmental risk assessment process, Select Bio Consult, LLC was retained to identify and review the available scientific literature on topics useful in assessing the risks to human and environmental health associated with the eukaryotic and photosynthetic green algae genus *Parachlorella*. This report contains a summary of the literature including citations used directly in the review, and a list of all other citations found, on the subjects of taxonomy, geographic distribution, environmental interactions, and reports of the impact of the genus on the environment.

Conclusions

Parachlorella spp. have been the subject of significant research using up-to-date taxonomic analysis. Reclassification of the genus and the species within it has been based on recent phylogenetic information using appropriate and modern technology, particularly nucleic acid sequence analysis, resulting in the ability to identify it precisely and accurately. Two species have been formally accepted in the phycology community and a third has been tentatively accepted, but physiology among the species appears to be very similar, and differences have not been well-characterized.

Parachlorella spp. have been found in a wide range of freshwater environments in many parts of the world, including California and its surrounding geographic region. *Parachlorella spp*. have broad tolerance to water conditions including extremes of temperature, pH, heavy metal contamination, salinity, and heavy nutrient load. Especially in water with heavy loads of carbon, nitrogen and/or phosphorus, *Parachlorella spp*. often successfully competes in the presence of a wide variety of other algal species, including certain cyanobacteria and the toxic substances they produce. However, harmful algal blooms (HABs) associated with *Parachlorella* have not been reported.

Enumeration of members within the genus, in the few cases where such detailed analyses has been carried out, are consistent with a genus that appears commensal or mutualistic rather than parasitic or competitive in a harmful way. *Parachlorella spp.* have been observed to appear in succession following cyanobacteria in a manner indicative of properties associated with beneficial water remediation. In fact, *Parachlorella spp*. have been studied directly for use in waste water remediation and have been added to systems where its effects are beneficial for recovery of wastewater.

No reports of adverse environmental effects associated with *Parachlorella* were found. However, it has been suggested that the ability of the algae to concentrate materials like heavy metals may result in indirect transfer of the toxic elements to species which use the algae as a primary food source. Specific documentation of such food-chain adverse effects were only found for *Parachlorella* in a couple of laboratory testing situations designed to use *Parachlorella* as the sole food source for multicellular species. No documentation of "natural" food-chain adverse effects was found. Moreover, the ability to concentrate heavy metals, herbicides, and similar potentially biohazardous materials is not unique to *Parachlorella spp.* nor does *Parachlorella* appear to concentrate such materials to a degree greater than any of a wide variety of other algae.

Parachlorella spp. are morphologically simple and have physiological properties in common with many other green microalgae, particularly to members of the *Chlorella* clade of organisms to which they are closely related phylogenetically. Also similar to their phylogenetic relatives, *Parachlorella spp*. are used as food for many other aquatic organisms including but not limited to potential scavengers, like Daphnia and other zooplankton, as well as for crustaceans. *Parachlorella* has been used as food in aquaculture of several shrimp species and includes subsequent use of the shrimp as human food.

No reports were found documenting toxins produced by *Parachlorella spp*., nor were reports found documenting infectiousness or adverse health effects in vertebrate animals, including humans. In contrast, feeding of *Parachlorella* to mice was reported to show beneficial effects, such as improved glucose tolerance and insulin sensitivity and a decrease of peripheral fat cell proliferation.

Parachlorella has been used as food directly for humans for many years. Until the recent taxonomic work, algae labelled as "chlorella" were identified as the source for human food, but recent work using nucleic acid sequence-based identification has clearly shown that some species used as human food are really *Parachlorella spp.*

In conclusion, the use and/or release of *Parachlorella* appears to be unusually well-documented as unlikely to cause harm. In fact, its presence is more likely to be beneficial than harmful.

Methodology

Searches were designed covering numerous scientific databases as described in Appendices included below in this report. The search criteria were broad in order to capture any results mentioning *Parachlorella* as currently classified in its own genus as well as detecting citations mentioning species currently within *Parachlorella* that were previously classified in the genus *Chlorella*. The original searches were supplemented with literature located as part of interpreting the initial search results when additional information was needed to provide contextual information for adequate interpretation.

As described in greater detail in Appendices below, 751 initial de-replicated citations were found. Two hundred thirteen articles met the search criteria for relevance to human and environmental health impact associated with the genus *Parachlorella* and were selected for detailed examination as full-text articles. Additional citations were added to provide additional contextual information for writing the report.

Results

Taxonomy

Identification of genus, species, strain

Algal taxonomy has continued to evolve with time, and these changes have accelerated in the past decade due to a shift in the technology used to evaluate relationships among genera and species. Earlier taxonomy relied heavily on morphology (size, shape, structure, color, etc.), first using light then electron microscopy, but its reliance has recently shifted to nucleic acid sequencing. Although morphology usually bears some relationship to physiological and biochemical properties, the simple visible characteristics of many single-celled algae do not provide sufficient insight into underlying properties and the differences between organisms. In this way identification strictly by morphology lacks accuracy. Moreover, many species can display different morphologies depending upon the environment it is living in. Genetic identification and classification by DNA sequencing, supplemented with traditional morphological approaches, has substantially reduced this ambiguity.

Since the advent of genetic bases for taxonomy, especially rapid nucleic acid sequencing and metagenomic analyses, more detailed phylogenetic and physiological insights into single-celled algae have become possible enabling more accurate assessments of environmental interactions. As a result, taxonomy has become more intimately linked to understanding the physiology and environmental interactions specific to an organism, which can then be extended to ecosystem-wide interactions. This serves to promote understanding of environment-dependent properties and genetics-dependent characteristics. Thus, detailed taxonomy provides additional insight into interactions between an organism with other organisms and with their environment.

Accurate taxonomy is necessary to correctly assess the scientific health, environmental and safety literature, particularly in cases where there have been substantial changes to taxonomic classification schemes. This process can be tedious and intensive but has gained in importance, particularly because of recent technological advances providing fundamental bases for accurate and detailed comparisons among and between organisms. Renaming taxonomic units – especially genera -- occurs, so it is necessary to determine which references using older taxonomic names are applicable to the genera and species of interest. Then, relevant documents published when the subject organism was classified under an older name should be located and included for consideration. Correspondingly, those reclassified or misclassified species that are removed from a genus of interest should be excluded from consideration.

The description of *Parachlorella* provided in this report includes consideration of literature regarding *Parachlorella* which were previously named differently, specifically as *Chlorella,* prior to the application of new nucleic acid sequencing-based phylogenetic analyses. Previous classification regimes for the genus *Chlorella* disguised a large number of distinct taxonomic groups with similar morphologic but distinct genetic features. One of these distinct phylogenetic genera, previously grouped within the classification of *Chlorella,* is now classified as the separate genus *Parachlorella*. Although considerable physiological and environmental overlap exists between the *Chlorella* and *Parachlorella*, there are ample distinguishing properties assignable to each genus which are important in understanding where *Parachlorella* are found and the role it plays in the environment.

Parachlorella is a genus within the higher-order taxonomic classifications in the Class Trebouxiophyceae and the Family Chlorellaceae. There are many morphologically very similar eukaryotic green microalgae within the class and family which, prior to taxonomy based on nucleic acid sequencing, led to misclassification of many organisms with similar microscopic appearance. Conversely, some morphological traits described for what were previously considered as unique genera, turned out to be the result of organisms similar in genetics and biochemistry being grown in different media or in different environments. Additionally, green algae with distinct morphologies have been shown by genetic analysis to be closely related phylogenetically to *Chlorella* and *Parachlorella*. Examples of common descriptions at Class and Genus levels are included below in considering the taxonomy of *Parachlorella* as currently defined. The scheme shown is based on information at Guiry, M.D. & Guiry, G.M. 2018. AlgaeBase. World-wide publication, National University of Ireland, Galway. (www.algaebase.org/search/species/detail/?species_id=P89bf83bea5658a68) The currently accepted taxonomic classification scheme is shown in Table 1.

Many members of the Family Chlorellaceae are difficult to distinguish morphologically. Taxonomy based on morphology alone, especially among planktonic coccoid green algae in the Class Trebouxiophyceae and the Family Chlorophyceae show that “morphology of these algae do not adequately reflect their phylogenetic position.”[1] Consequently, nucleic acid sequencing, especially 18S rRNA and internal transcribed spacer regions (ITS2), are widely accepted as necessary for adequate phylogenetic classifications of many algal and other species, especially members of the Chlorellaceae Family.

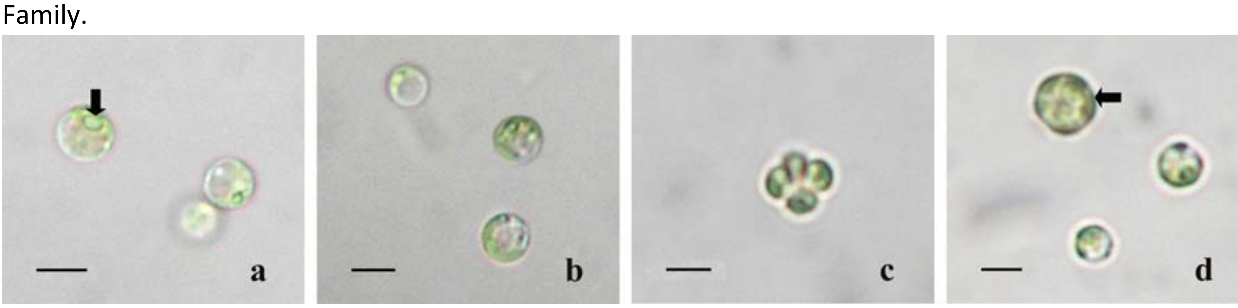


Figure 1. Photomicrograph of *P. kessleri*. a. Vegetative cells with one pyrenoid (arrowhead); b. Cup-shaped chloroplast; c, d. Sporangium with four or six autospores. *P. kessleri* identity was confirmed using 18S rRNA and ITS sequences.[2]

Kingdom – Plantae; Subkingdom – Viridiplantae Infrakingdom – Chlorophyta Phylum – Chlorophyta; Subphylum – Chlorophytina Class – Trebouxiophyceae Swimming cells with one or two pairs of flagella, without mastigonemes; basal bodies with four microtubular rootlets in cruciate arrangement, including a multilayered structure, and a smaller root, alternating between two or more microtubules; basal bodies with prominent rhizoplast, cruciate, displaced counter-clockwise; counter-clockwise basal body orientation; closed mitosis with metacentric spindle, semi-closed mitosis, cytokinesis with phycoplast; asexual reproduction by autospores or zoospores; sexual reproduction reported; lichenose and free-living forms; osmotrophy and autotrophy. Order – Chlorellales Family – Chlorellaceae Genus – Currently 56 genera (including *Chlorella* with 44 species). The genus *Parachlorella* has two recognized species and one species with tentative taxonomic status. Species *P. beijerinckii* (type species) *P. kessleri* (previously *Chlorella kessleri*) *P. hussii* (not yet formally recognized)

Table 1. Classification of eukaryotic green microalgae in the taxonomic Class Trebouxiophyceae. The figure is based on information in AlgaeBase (Guiry, M.D. & Guiry, G.M. 2018, as above).

Nevertheless, especially when reinforced using nucleic acid sequence data, morphology can be useful in distinguishing species. Table 2 provides descriptions of the morphological properties unique to *Parachlorella* and specific for each of the two formally recognized species. Additionally, closely related species as determined using nucleic acid sequencing, which were previously classified as markedly different than members of the *Chlorella* genus based on morphology, are included in the table.

Table 2. Morphological features of Parachlorella and genetically-related organisms

Species	**Morphology**
Chlorella spp.	Cells spherical, subspherical or ellipsoid, single or forming colonies with up to 64 cells, mucilage present or absent. Chloroplast single, parietal, pyrenoid present, surrounded by starch grains. Reproduction by autospores, zoospores lacking. Autospores released through disruption of mother cell wall. Daughter cell can remain attached to remnants of mother cell wall and form colonies with mucilage envelopes. Planktonic, edaphic or endosymbiotic.
Parachlorella spp.	Solitary planktonic or edaphic globose or egg-shaped cells, sometimes with a thin, membranous gelatinous coating; parietal chloroplast with broadly ellipsoidal pyrenoid covered by starch grains; reproduction via 2, 4, 8, or 16 autospores;

Table 2. Morphological features of Parachlorella and genetically-related organisms	
	distinguished from other genera in the family by 18S rRNA and ITS2 nucleic acid sequences.
Parachlorella beijerinckii	As above with cells 2.5-5 x 3-8 µm with a 2-4 µm think gelatinous coat; vegetative cells are spherical or ellipsoidal with 5-8 µm diameter; single pot- or saucer-shaped chloroplast with broadly ellipsoidal pyrenoid covered with 2, 3 or 4 large cup-shaped starch grains; one or two thylakoids traverse the pyrenoid; reproduction by 2, 4 or 8 autospores sized 2.5-3.5 x 3-4.5 µm which were liberated by a broad opening in the mother cell leaving a cup-shaped empty mother cell wall remnant; cells surrounded by amorphous mucilage; electron microscopy revealed a single-layer cell wall; species differentiation by nucleic acid sequencing.
Parachlorella kessleri	In contrast to *P. beijerinckii*, *P. kessleri* has a mantle-shaped chloroplast and no mucilaginous coat.
Parachlorella hussii	Solitary, planktonic cells with, oval young cells and spherical to slightly oval adult cells 4.5–6.5 (7.5) µm; adult cells are surrounded by a gelatinous coat 1–3 µm thick; a single, parietal, cup-shaped chloroplast and a broadly ellipsoid pyrenoid, which is covered by two starch grains; reproduction by autosporulation with 2, 4 or 8 autospores; species differentiation by nucleic acid sequencing.
Closteriopsis acicularus (in *Parachlorella* clade)	Long needle-shaped with 2 to 6 starch-covered pyrenoids.
Dicloster arcuatus (in *Parachlorella* clade)	Two-celled coenobia with elongated ellipsoidal cells and long pointed apices; a single parietal chloroplast with two pyrenoids.

The morphological features in the table are for cells grown in aerated suspension cultures. However, growth on agar resulted in loss of the "typical" morphology. Morphological changes in *Chlorella* clade organisms included modifications such as loss of spines or coenobia with some species assuming a more spherical shape resembling the "typical "shape of true *Chlorella* species.[3] The results indicate the difficulty in finding stable morphological features in different growth conditions and in defining phylogenetic relationships based on morphology alone.

Genetic Evidence for Taxonomy

The importance of accurate taxonomy and an awareness of taxonomic history for a particular genus includes the need to be able to find literature describing a particular genus or species as the designations assigned to an organism change over time. In the case of the genus *Parachlorella* the changes are largely based on improved methods for identification of the genus and species within it.

The major technological change in taxonomy since the mid-2000s has been the general application of nucleic acid sequencing, especially sequences coding for 18s rRNA and internal transcribed spacer (ITS) regions. Fortunately, in *Parachlorella*, the species names have been retained, i.e. *beijerinckii* and *kessleri*, which were previously classified as *Chlorella beijerinckii* and *Chlorella kessleri*. One potentially confusing problem is the Gram-positive anaerobic bacteria *Clostridium beijerinckii*, for which a large body of literature exists and for which the same abbreviation, C. *beijerinckii*, is frequently used. However, since *Clostridium* and *Chlorella* are markedly different organisms with different properties, the relevance of a particular publication is readily determined from titles and abstracts.

Note that ITS2 sequences are considered as more highly variable than are the 18S rRNA sequences. As was noted in the work leading to the separation of *Chlorella* and *Parachlorella* as separate genera, "morphological criteria traditionally used for classification (e.g. spines, mucilaginous envelopes or the formation of colonies or coenobia) are burdened with a high degree of phenotypic plasticity. These plastic traits represent adaptations to environmental factors in order to optimize floating in the water column and to resist grazing pressure."[3] Krienitz et al.[3] also mentioned that a variety of algae have been described as Chlorella-like, based on the minimal structure of "… a single nucleus, chloroplast, and mitochondrian, some peroxisomes and vacuoles." The authors suggested that the minimal structure has the adaptive advantage of the coccoid morphology in an ecosystem. Different ecosystems may favor similar morphology but different physiology and biochemistry. Consequently, considerable variety may be hidden within simple and similar morphology. Genetic analyses, including the recent separation of *Chlorella* and *Parachlorella* into distinct genera, support this view of complexity within common morphology. Also, based on use of 18S rRNA sequences, algae with other than coccoid morphology have been found to belong in the clade of true *Chlorella sensu stricto*.

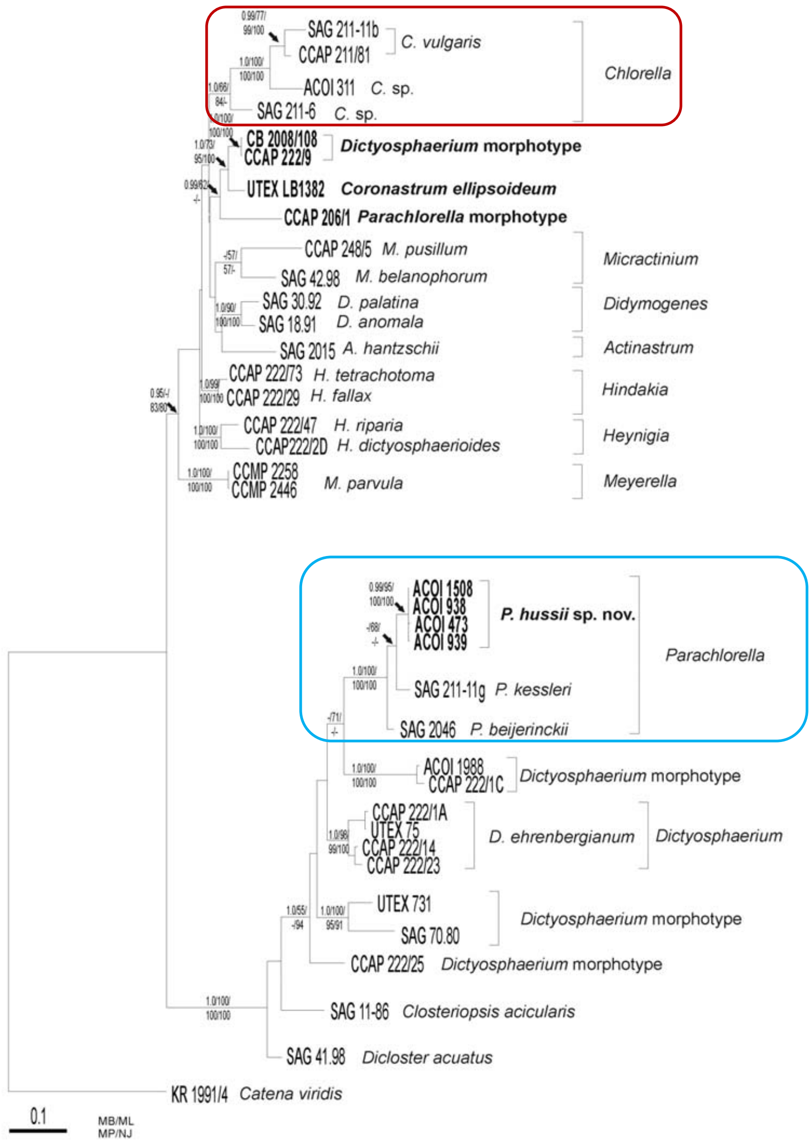
Bock, et al.[4] proposed another unique species to be included within the genus *Parachlorella*, *Parachlorella hussii*, which is listed in AlgaeBase as having uncertain taxonomic status. The basis for the classification is consistent morphology with other *Parachlorella* and both 18S rRNA and ITS2 sequence data. The phylogenetic tree including the newly proposed species within the context of *Chlorella* and *Parachlorella* genera is reproduced from the publication (Figure 1). The authors described *P. hussii* as more closely related to *P. kessleri* than to *P. beijerinckii*.

A strain isolated downstream from the Fukushima Daiichi Nuclear Plant was viable at high temperatures and resistant to high and low pH, i.e. 3-11, and capable of growth in fresh or sea water. 18S rRNA sequences showed a close relationship to *P. kessleri*, but actin mRNA indicated differences. A new species, *P. binos*, was proposed. Note: the new species does not appear to have been accepted and was found only in literature via direct citation of the original paper.[5] The species name does not appear in AlgaeBase (http://www.algaebase.org/).

Numerous new *Parachlorella*-related species have been identified recently, predominantly by use of nucleic acid sequence information. Although no new *Parachlorella* species have been identified other than those named above, new genera have been described within both the *Chlorella* and *Parachlorella* clades.[6-11] Interesting relationships with other organisms have been observed, such as shared ancestry across taxonomic orders[10] and symbiotic living with *Paramecium*.[12] Morphological data also contributes to understanding taxonomy,[5, 11] although use of morphology is declining, as is use of biochemical markers such as analysis of cell wall components.[13-14] Frequently, the link expected between details of genetics and expression of particular and sometimes genus- or species-specific structures is supported.

One of the difficulties in classification of *Chlorella, Parachlorella,* and closely related genera, as well as difficulty distinguishing among various genera and species, was that the formation of autospores is uniform across all groups. Distinctions based on autospore formation among Chlorellaceae are difficult in contrast to many other algal families where differences in autospore formation have been used in defining taxonomic groups. However, one of the features used before widespread use of nucleic acid sequencing was that the ultrastructure and chemical constituents in autospores differ between *Chlorella* and *Parachlorella*. The differences were not evident at the light microscopic level and the ultrastructural differences were reported in 2005, after phylogenetic distinctions between the genera had been reported using nucleic acid sequencing.[15]

Figure 1. Phylogenetic tree of Chlorella and Parachlorella.[4] *Chlorella* clade outlined in red and *Parachlorella* clade outlined in blue.



Distribution of Species

Regional Distribution

Two areas of the world have the most reported localizations of *Parachlorella*; Europe, especially in or near the Czech Republic, and eastern China. The former is the result of intensive study of *Parachlorella* in laboratories in the Czech Republic for more than 50 years. The latter is probably the result of the use of *Parachlorella* as a food source, as noted in further detail below. There is no obvious reason to suspect that *Parachlorella* is restricted to the locations in which it has been reported so far. In fact, recent work found *Parachlorella* in California and Mexico in addition to other locations in the Western Hemisphere,[16] which are included on the map below.

ii. Occurrence of Species

Figure 2. Locations where *Parachlorella* spp. have been isolated. See table in Appendix for more details.

Environmental Factors

Water quality tolerances

The genus *Chlorella*, prior to recent taxonomic reclassification, was described as "among the best-studied unicellular green algae".[14] Dating back to the early 20th century, *Chlorella* served as model organisms in plant physiological and biochemical studies including fundamental research on photosynthesis and nitrate reduction. Moreover, members of this group of algae have been used as human food and for waste treatment and water recovery for many years. Work with "*Chlorella*" is now known to have included work with newly defined genera, specifically including *Parachlorella*. The results and long-term use of these eukaryotic green single-celled microalgae infer a degree of safety and limited adverse effects on the environment. Nevertheless, it is important to consider details of previous studies with regard to what has been studied and the outcomes predicted based on those studies.

Growth requirements for what was then described as *Chlorella kessleri* (now *Parachlorella kessleri*) were presented as: able to reduce NO^{3-}, pH optimum of 2.5-3.0, and growth in 1-2% NaCl at optimum temperature of 34-36°C, very similar to what are now prototypical species of *Chlorella*.[14] The larger group, i.e. classification of *Chlorella* before *Parachlorella* was separated, were referred to as "green balls" which had been found in freshwater, marine, and soil habitats.[1] Widespread use of *Chlorella*-like algae, then and now, apparently results from their relatively simple growth requirements. For example, *Chlorella* and *Parachlorella* of many species grow readily in Bold's Basal Medium, which contains phosphate, nitrate, sulfate, borate, K, Ca, Mg, Na, Zn, Mn, Mo, Cu, Co, and Fe all in low concentrations at pH 6.8. No energy sources are needed, if the cells are grown in light of appropriate wavelengths, although the cells will use glucose as an energy source in mixotrophic growth. Such simple growth requirements are consistent with the broad distribution of the *Parachlorella* genus that has been observed. However, broad distribution also requires that an organism be tolerant of a variety of environmental conditions. Numerous studies summarized below indicate the degree to which this is true for *Parachlorella*.

Ion effects

Numerous metal and other ionic species have been evaluated for effects on growth of *Parachlorella*. Of 4 species/genera tested, *Parachlorella* had the greatest apparent affinity and capacity for cadmium absorption.[17] The other genera tested were *Nannochloropsis*, *Spirulina*, and *Scenedesmus*, all of which have been used as human food.[17-18] *Scenedesmus* is also a eukaryotic green microalga like *Parachlorella.* Over the range of "environmental concentrations (0-32 µg/L)" of Cd, toxic effects were observed at concentrations as low as 3 µg Cd/L and pronounced effects on growth, morphology, size and physiological state were observed at 8 µg/L.[18-19] *P. kessleri* is more sensitive to Cd than is the *Chlorella* type-species, *C. vulgaris*.[20] Toxicity of several metals was tested using *P. kessleri* and another freshwater algae, *Raphidocelis subcapitata*, a green microalgae frequently used as a bioindicator for nutrients and toxicants in water.[21] Hg showed the greatest toxicity for both algae, but *P. kessleri* was more sensitive in general than was *R. subcapitata*.[22] *Parachlorella kessleri*, *Scenedesmus quadricauda*, *S*. *subspicatus* and *Raphidocelis subcapitata* were tested for Cd toxicity, which was the most toxic metal for all four algae including *P. kessleri*. Silver sorption by *P. kessleri* was found to be predominantly the result of adsorption to the surface. Silver removed from solution by living cells was released back into the solution, although there was evidence of silver bioreduction over time.[23] Effects of sublethal concentrations of copper were tested on three strains of green microalgae under autotrophic and mixotrophic culture conditions. Bleaching and lipid peroxidation were reported in *P. kessleri* cultures grown in the presence of sublethal Cu concentrations. Both were more pronounced in the absence of organic carbon, i.e. under autotrophic conditions.[24] Growth inhibition by organotin compounds was greater in *P. kessleri* than in *Euglena gracilis*. The cells were tolerant of inorganic tin salts but tributyltin and tributyltin oxide were growth inhibitors.[25] The results show that *Parachlorella*, particularly the species *P. kessleri* which has been tested directly, is sensitive to contamination by heavy metals more than are a number of other algae. Thus, although *Parachlorella* and other eukaryotic green microalgae can concentrate some toxic metals, the greater degree of sensitivity for *Parachlorella* may indicate that it is less likely to grow in metal-contaminated waters than are some other eukaryotic green microalgae used as food.

Severe environments

P. kessleri was isolated from a natural mesothermal acidic pond in Argentina and was present at high density. The waters have a high concentration of sulfuric acid. The results indicate that the species can be adapted to live in extremely acidic conditions.[26] *Parachlorella* algae isolated downstream from the Fukushima Daiichi Nuclear Plant was viable at high temperatures and resistant to high and low pH (3-11) and capable of growth in fresh or sea water.[5] *P. kessleri* was one of two species isolated from a coal-fired thermoelectric plant in Brazil. Growth rates were measured in several concentrations of CO_2 with significant biofixation in concentrations of 6% and 12% CO_2 and useful values in 18% CO_2. The authors claimed that the growth properties are consistent with utility for biofixation of CO_2 in thermoelectric plants.[27]

Other stresses and tolerances

<u>Natural waters</u>

E.C. Kessler, the eponym for the strain *P. kessleri*, tested physiologic properties of the algae and found, "*C. kessleri* (7 strains) shows a rather low rate of fermentation. Formic acid, CO_2 and H_2 were identified." Kessler reported on glucose fermentation in 87 strains of green microalgae then-identified as *Chlorella*. These included species now classified as *Parachlorella*. Fermentation products were species-specific, e.g. high levels of glycerol were found only in the most salt-tolerant species,[28] which do not include *Parachlorella spp*. Assays to evaluate algal growth potential (AGP) for wetlands along the Danube River[29] was developed beginning in the late 1990s and has been used since then to evaluate AGP.[29-34] The choice of *P. kessleri* as a test species for these waters, including the Danube River and the Drava River, which is a major Danube tributary, was probably because the species has been found there in addition to its simple growth requirements.[29, 31] *P. kessleri* was used as a test species for evaluating water quality to indicate growth conditions resulting from nutrient and toxic interactions. Note, however, that the testing was not designed to test toxic effects generated by the *Parachlorella* species. Rather, the results show the effect of different sources of water on potential for growth of *Parachlorella*. Addition of phosphate to water from the Draga River improved growth of *P. kessleri*. Heavy metals and toxic substances from pollution of the river were present in the waters used for testing.[29, 35] Samples were taken over months in spring, summer and early fall and included flood and low water conditions as sources of water from the Danube River watershed.[34, 36] "During the whole investigation period, nitrates control the eutrophication in Kopacki Rit".[29] Growth limitation seemed to track most with nitrogen rather than phosphorus. *P. kessleri* was consistently used as the test organism for waters from natural sources. Nitrogen and phosphorus limitations resulting in the greatest growth-limiting results varied by season.[33, 37] Waters with a nitrogen/phosphorus (N/P) ratio of 16-22 supported higher growth than those with N/P < 14. Water levels and flooding affected nutrient levels in the water.[34] The authors discussed the importance of understanding temporal and spatial properties of the flood plain hydrology to understand nutrient potential of the waters in and along the Danube. Horvatic et al.[31] used the *P. kessleri* assay to determine algal growth potential in Lake Sakadas, which is part of the Danube River watershed. They followed phytoplankton successions in the lake, which included month-long periods dominated by diatoms, Chrysophyceae (Golden Algae), a depression of phytoplankton followed by an algal bloom of cyanobacteria, a Synura (Ochrophyta) bloom, and Euglenophyceae dominance. Although the water clearly supported growth of *P. kessleri*, which was directly tested, it may be of significance that the dominant species were algae other than Chlorophyceae, i.e. other than *P. kessleri*.

<u>Waste waters</u>

Green microalgae have been of significant interest for wastewater treatment for a number of years[38] with the Chlorophyta *Chlorella* spp. and *Scenedesmus* spp. often mentioned as interesting.[39] *Parachlorella*, which is closely related phylogenetically, has also been mentioned, albeit less frequently.[40] The growing literature indicates interactions between the environment and *Parachlorella*.

Microbial diversity in open wastewater ponds was recently examined.[41] Microalgae in natural algal wastewater ponds included Chlorellaceae, specifically *Parachlorella,* among other green microalgae of the same taxonomic Family as dominant members of the diverse algal community. Cyanobacteria were present seasonally and were attributed to cyanobacterial blooms from influent wastewater streams. Cyanobacteria decreased "giving way to Chlorellaceae and Scendesmaceae, which became dominant during spring and summer months." At higher temperatures, i.e. 35°C, *Parachlorella* and related genera "showed highest specific growth rates" while other green algae were dominant when temperatures were 20° to 25 °C. The authors stated "… these results show that microalgal diversity helps in tolerating adverse environmental conditions, thereby maintaining a critical microalgal-bacterial ratio …", in the case described by these authors, for treating wastewater. Identification of relevant species, such as

Parachlorella, included morphological as well as genetic analysis. Diversity of microalgae present in such systems is important, and in this case the role of *Parachlorella* was specifically described.[41] It is informative to recognize that *Parachlorella* was a naturally occurring genus in this case and was considered as an important microalga for maintaining the effectiveness of the wastewater treatment pond as part of a diverse microalgal population.

P. kessleri and five other species from 4 genera, including 2 *Chlorella* species, were adapted to growth in secondary-treated municipal wastewater during an 8-week period.[42] The authors stated: “A wastewater environment can be particularly toxic to eukaryotic microalgae”.[42] *P. kessleri* was one of two species that showed the greatest ability to acclimate to growth in wastewater. “… in particular, non-acclimated *P. kessleri* was as tolerant as many other acclimated species.” *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Parachlorella kessleri* were cultivated in batch experiments to test for nutrient removal from wastewater.[43] Nitrogen and phosphorus removal were greater than 90% for all species. “All four species tested have high potential for removing nitrogen and phosphorus from urban wastewater to levels even lower than the most restrictive currently imposed …” based on European Commission Directive 98/15/EEC. Additionally, *P. hussii* was isolated from secondary wastewater and was one of the most resistant to oxidative stress (H_2O_2) among five indigenous chlorophyte microalgae strains isolated from a municipal secondary wastewater effluent tank. The stress resistance apparently contributed to the ability to grow well in raw waste water. The resistant algae were able to produce high levels of biomass and lipids in the wastewater effluent. “These results demonstrate the potential of these two strains for future biofuel applications coupled to wastewater remediation and highlight the importance of oxidative stress tolerance as a key indicator of efficient wastewater growth.”[44] The results indicate the high degree of adaptability of *Parachlorella* and related eukaryotic green microalgae genera to this harsh environment.

Green microalgae of a wide variety of types have also been tested in and considered further for use in heavy metal remediation in wastewater.[45-47] Although *Chlorella* and *Scenedesmus*, among other types of microalgae, have most frequently been tested in heavy metal remediation, *Parachlorella* have also been tested. *P. kessleri* was among six Chlorophyta species tested and was one of the most resistant to toxicological challenges by Cr, Cu, and Zn and by the herbicides oxyfluorfen, pendimethalin, and atrazine.[48] Resistance to Cd toxicity and accumulation of the metal was greater in *Chlorella* (*C. luteoviridis*) and *Parachlorella* (*P. hussii* and *P. kessleri*) strains that had previously been adapted to growth in wastewater. The wastewater-adapted strains also showed increased tolerance to Cu, Al, and Zn.[49] The chloroacetanilide herbicide, S-metolachlor (S-meta), was added to cultures of *P. kessleri* and growth and reactive oxygen species (ROS) content was observed. Although inhibited by S-meta, growth continued at a reduced rate. The authors concluded that the still-dividing damaged algal cells “could represent an important environmental toxic factor that might affect higher organisms in the food chain.”[50] Accumulation and metabolism of the herbicide isoproturon was studied in *P. kessleri* and *Anabaena inaequalis* as a function of pH, pesticide concentration, and incubation time. Metabolism of the pesticide was nearly identical between the two algae. Despite the phylogenetic differences between the prokaryotic and eukaryotic algae chosen for this study there are apparently significant similarities for metabolism of isoproturon in common between them.[51] “Polybrominated diphenyl ethers (PBDEs), widely used as flame retardants, are persistent contaminants which cause adverse effects to environments and pose health risks to human.” *Chlorella*, *Parachlorella* and strains from other algal genera were recovered from wastewater and shown to be PBDE-tolerant. Although *Chlorella* species were more effective at removing PBDEs, *Parachlorella* strains were also able to remove the compounds from solution.[52] Thus, *Parachlorella* can accumulate toxic entities, such as heavy metals and herbicides

from the environment, when the materials are present. However, in addition to accumulation, bioremediation by *Parachlorella* may occur via sequestration or metabolism.
The accumulation of toxic materials may provide a way to pass the toxins through the food chain, but no cases of such food-chain related events were found in the literature. Moreover, consideration of the results appears to be most accurately interpreted as a reaction of the microalgae to their environmental conditions. No evidence was found of *Parachlorella* as causative in generation of toxins or harmful materials. Practical application of *Parachlorella* environmental properties is most often considered as potentially beneficial rather than threatening. Also, as noted in citations above, many species of microalgae have been shown to accumulate heavy metals and herbicides, so *Parachlorella* does not appear to present a unique hazard in this regard.

Reproductive requirement

Reproduction of *Parachlorella*, as for *Chlorella*, is asexual by means of formation of autospores.[14] Conditions describing triggering of autospore formation were not found in the available literature or in textbooks of phycology.

Effects on biological co-inhabitants

<u>Succession and pond crashes</u>:
Allelopathic effects of cyanobacteria on algae were evaluated to observe the effects of microcystins (MC) and cylindrospermopsin (CYN) on algae, including *P. kessleri*. Growth rate and photosynthetic pigments were not affected by extracts from either of two MC and CYN producers. No allelopathic effects were observed under the conditions of the experiment consistent with a high tolerance by the eukaryotic green microalgae for cyanobacterial toxins.[53] As mentioned above, *Parachlorella* was considered as an important part of a natural and diverse microalgal population for maintaining waste water ponds,[41] which included recovery from cyanobacterial blooms in influent waters. The same work showed that *Parachlorella*, as well as other Chlorellaceae, was vulnerable to predation by Daphnia sp., which could have adverse effects on pond microbial stability.[41] In such cases pond biodiversity was important for providing a collective adaptability to the pond biodiversity. Another predator reported for Parachlorella was the rotifer *Brachionus calyciflorus*.[54] The results from these studies indicate that there are natural predators to *P. kessleri* which can greatly suppress growth of the algae.

<u>Shrimp</u>:
Litopenaeus vannamei (Pacific white shrimp or king prawn) is a type of shrimp grown in ponds in aquaculture. Among 16 species of microalgae*, P. kessleri* was found as one of the dominant species in the aquiculture ponds during “mid-phase”, i.e. around 33 days in the 60+ day growth cycle of the shrimp-producing ponds. Green algae, specifically *Parachlorella* in this study, was associated with good breeding among and growth of the shrimp. The authors discussed likely effects of the microalgae populations with the appearance of *Chlorella,* now known to include *Parachlorella*, being desirable in contrast to cyanobacteria for maintaining a stable environment for the growth of shrimp. The discussion also mentions that the green microalgae appear to compete with dinoflagellates, which have low food value for the shrimp, and with red tide-like dinoflagellates and associated toxins. Green microalgae and diatoms appear to provide absorption of “harmful substances”. The health of pre-larval shrimp, as in the early stage of shrimp growth, was particularly important for the survival of shrimp increasing the efficiency of shrimp production. The work *mentions* “*C. kessleri*” as one of the green algae species with dominance in the mid-growth period.[55] Effects of a dry powdered microalga *Parachlorella kessleri* (KNK-A001) fed as a supplement to two shrimp species found that dietary supplementation of 0.005% KNK-A001 showed a tendency to improve growth performance of pacific white shrimp, *Litopenaeus*

vannamei.[56] Furthermore, kuruma shrimp, *Marsupenaeus japonicus*, fed a diet containing 0.05% KNK-A001 showed a significantly higher survival rate than shrimp grown without KNK-A001. Increased hemocyte cell density was observed in the hemolymph obtained from shrimp fed 0.05% KNK-A001, and superoxide anion-producing activity of the hemocytes was also increased compared to control cultures. The results suggest beneficial effects by the presence of *Parachlorella* on the shrimp species.

Bioaccumulation of toxic substances

P. kessleri was exposed to hexachlorobenzene (HCB) for three days before being used as food for *Chasmagnathus granulatus*. After more than two weeks, the crabs showed accumulation of HCB in hepatopancreas. Enzyme inhibition and oxidative stress were observed in crabs with accumulated HCB. The data indicated that the HCB was accumulated by the algae and transferred to the crabs feeding on them. Morphological/pathological effects were also seen in the crab hepatopancreas.[57] The results show that the bioaccumulation of some substances by *Parachlorella spp*. may have undesired side effects in the form of concentration of a toxic substance when the algae were used as a food source by the crabs.

Daphnia magna is a zooplankton crustacean sometimes used as an indicator organism for aquatic toxic effects. Direct exposure of the daphnia to the herbicide pendimethalin was compared to treatment of *P. kessleri* with pendimethalin as food source for the daphnia. Mobility of the daphnia was observed to determine effects of the herbicide. Addition of untreated *P. kessleri* to *Daphnia magna* improved motility indicating that *P. kessleri* was being used as food for the crustacean. Daphnia exposed to pendimethalin showed decreased mobility whether treatment was directly on daphnia or added to the *P. kessleri* before addition to the crustacean. However, motility decreased more when *P. kessleri* was treated with the herbicide without direct exposure of the daphnia than if the daphnia were exposed directly to the herbicide. The greatest decrease in motility was observed when both the daphnia and *P. kessleri* were exposed to pendimethalin. The results indicate that *P. kessleri* may concentrate pendimethalin thereby resulting in greater effects of the herbicide than resulted from direct exposure of the daphnia. However, interpretation of the study is complicated by limited replication and a lack of statistical analysis of the results. Nevertheless, the results are consistent with possible concentration of the herbicide by *P. kessleri* leading to greater exposure of an aquatic zooplankton than would occur in the absence of the algae.[58] Another study found that low concentrations (< 0.1 ppm) of Cu inhibited rotifer (*Brachionus calyciflorus*) growth but did not affect *P. kessleri*. However, a higher concentration of Cu (1.5 ppm) was needed to inhibit rotifer growth, since the algae bound Cu removing it from solution. Fatty acid methyl ester extraction, i.e. the material used for biodiesel productions, was not affected by the Cu.[59]

Bioaccumulation by *Parachlorella* of toxic substances is not unique. Many algae, as well as other types of organisms concentrate hydrophobic organic water contaminants[60] such as chlorobenzenes.[61-62] Consequently, the bioaccumulation noted above for *Parachlorella* should not be considered as a unique property of this genus.

Symbiosis:

Species-specific ability of *Chlorella* strains (Chlorophyceae) to form stable symbioses with *Hydra viridis* was evaluated. Of 15 algae then classified as "*Chlorella*", six, including *P. kessleri*, formed stable symbiotic relationships with the freshwater polyp *Hydra viridis*. Symbiosis appeared to correlate with ability to grow at or below pH 4.0.[63]

iv. Bloom reports

No bloom reports, including those for harmful algal blooms (HABs), were found despite the presence of *Parachlorella* in many natural water environments. Likewise, the closely related genus *Chlorella* did not appear to be associated with algal blooms or HABs.

Evidence of toxicity, infectiousness, adverse health effects in vertebrates

Several studies have reported on the use of "*Chlorella*", as food and/or as a medicament. As noted elsewhere in this report, the currently accepted taxonomic classification schemes place *Parachlorella* as a close relative to the species currently formally accepted as *Chlorella*. Moreover, past works and commercial companies have not used refined taxonomic classification and many studies nominally declared as *Chlorella* are genuinely *Parachlorella*. In the remainder of this section, studies that explicitly mention *Parachlorella* are summarized.

Several reports described tests of *Parachlorella* in animal feeding studies to evaluate claims of potential benefit from the algae. "Powderized *Parachlorella beijerinckii* (BP) and its hot water extract are believed to be useful for preventing common diseases such as hypertension, arteriosclerosis, and hyperlipidemia. The present study investigated how *Chlorella* components influence common diseases in obese mice and rats on a high-fat diet."[64] *P. beijerinckii* was used as the source for the powder and extract. C57Bl/6 male mice and Sprague-Dawley rats were used. One group from each species was fed a high fat diet, one group was fed a low-fat diet, and a third group was fed a high fat diet with BP supplemented. "Results: BP administration had no effect on high-fat diet-induced obesity. However, compared with the high-fat diet group, the group with added BP had improved glucose tolerance and insulin sensitivity and inhibited the hypertrophic growth of visceral fat cells. In addition, the BP group had improved serum adiponectin, leptin and monocyte chemoattractant protein-1 (MCP-1) levels. The MCP-1 expression level in epididymal fat was decreased in the BP-treated group." Administration of the hot water extract reduced the amount of peritesticular fat and serum triglyceride (TG) levels. The results suggest that the antihyperinsulinemic effects of BP are due to the modulation of adipose tissue hypertrophy and adipocytokine secretion. The hot water extract inhibited the accumulation of visceral fat and serum TG. In another study, "chlorella extract" consisting of a hot water extract of *Parachlorella beyerinckii* (sic) which was "… previously identified as Chlorella vulgaris …", was fed to male and female rats as 0.2, 1, or 5% of their diet for 13 weeks. Body weights, urinalysis, clinical blood chemistry, and organ weight did not differ among the rats regardless of the amount of the algal extract fed. The authors declared a no adverse effect level (NOAEL) of 5% of the diet containing the algal extract corresponding to a daily intake of more than 3 g/kg body weight.

Medicinal properties associated with *Parachlorella* and extracts from it have been proposed. Exopolysaccharides extracted from *P. kessleri* were observed to have significant bioactivity in inhibiting colon carcinoma cell growth in culture.[65] A hot-water extract from a strain of *P. kessleri* showed immunostimulatory activity, observed as increased natural killer cell activity in mice and as a protective effect on virus-infected model shrimp.[66]

The ability of *Parachlorella* feeding to eliminate toxic metals in mice has been explored. *P beijerinckii* powder was fed to mice and elimination of tissue methylmercury was observed, especially via elimination into urine and feces. The improved elimination did not change glutathione metabolism, although tissue Hg levels are reported to be closely related to glutathione metabolism. Thus, the *Parachlorella* powder may be useful for eliminating methyl-Hg from tissues.[67]
Dried powder from outdoor grown *P. beyerinckii* (apparently *beijerinckii* ?) was fed to mice (100 mg) with or without lead as lead acetate at 20 or 40 mg/mouse.[68] Fecal excretion of Pb was greater in mice fed the algal powder than in those not fed the powder. Also, lead levels in blood, liver, and kidney were

reduced 24 hr. after Pb administration in mice fed algae. Concentrations of Pb were 48-69% lower than in control mice. The results suggest that feeding of the algae may be useful in treating Pb exposure in humans.

Interactions with the environment (biochemical cycling)

Although not all algae necessarily are useful for wastewater remediation, members of the *Chlorella* and *Parachlorella* clades have been tested for their utility in wastewater remediation in several studies. Since this indicates properties of the algal interaction with the environment, results from the studies involving *Parachlorella* are described below.

Municipal wastewater treatment

Chlorella (*Parachlorella*) *kessleri* and *Chlorella protothecoides* were cultivated in highly concentrated municipal wastewater. Various light intensities were used, and corresponding biomass accumulation was determined. "Both species were capable of wastewater nutrients removal under all lighting conditions with high removal efficiencies".[69] Secondary-treated municipal wastewater and sludge liquor from dewatering of activated sludge were used to grow indigenously isolated algae, including *P. hussii* and several other algae. *Parachlorella hussii* was one of two strains tolerant to the wastewater liquor with a concentration of 25% liquor providing the best growth, biomass productivity, and nutrient removal values. *P. hussii* was capable of growth all year including in autumn and winter but with strongest growth, productivity, and remediation characteristics in the summer and spring. Monoculture growth was maintained with no significant contamination or culture crash, demonstrating the robustness of the strain for wastewater cultivation in a northern European climate.[70] In another study the growth characteristics and nitrogen removal capacity of five species of green algae were compared.[71] *P. kessleri* had the highest biomass accumulation compared to any of the other species and was also best for nitrate removal. Secondary urban wastewater was used to culture several types of algae, including *P. kessleri*, and biomass productivity was higher in the wastewater than in synthetic media.[72] "The freshwater microalgae species *C. kessleri* and *C. vulgaris* removed N and P almost completely (N 95%) from urban wastewater." *P. kessleri* was reported to be a suitable algal species for remediation of municipal wastewater because it grew quickly in undiluted wastewater and produced significant biomass. The algae also efficiently removed nutrients from the wastewater, e.g. ~80% of total nitrogen, >90% of total phosphorus, 60-80% of Fe, Mg, and ammoniacal nitrogen, and 60% or more of chemical and biological oxygen demand and alkalinity.[73] Supernatant from activated sludge wastewater was used to grow a locally isolated algae identified as *P. kessleri*.[74] Effect of the light-dark cycle was minimal, although there was sensitivity to pH. The strain was highly effective at removing total nitrogen, phosphorus, and chemical oxygen demand (COD) with a high growth rate and biomass productivity using the wastewater as culture medium. Thus, the results from numerous studies showed that the presence of *Parachlorella* can have beneficial effects when present or added to wastewater for water remediation.

Treatment of other wastewaters

The removal of ten target metals of environmental concern (^{53}Cr, Mn, Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, As, ^{88}Sr, ^{95}Mo, and Ba) was tested from oil sands tailings pond water. "The organism responsible for removal was found to be an indigenous green micro-alga isolated from cyclone overflow water (Syncrude) and identified as *Parachlorella kessleri* by sequencing of the 23S rRNA gene. *P. kessleri* grew in tailings pond water samples taken from two oil sands operators (Syncrude Canada Ltd. and Albian Sands Energy Inc.), and enriched with low (0.24 mM NO_3 and 0.016 mM $PO^{-3}{}_4$) and high (1.98 mM NO_3 and 0.20 mM $PO^{-3}{}_4$) concentrations of nutrient supplements (the most realistic scenario)." High concentrations of nitrogen and phosphorus adversely affected removal of Co, ^{60}Ni, As, ^{88}SR, and Mo.[75] A two-stage anaerobic bioreactor was used to process agro-industrial wastes from sources such as end-of-life dairy products, pig manure, and slaughterhouse wastes. A liquid supernatant was used from the treated waste as culture medium for algae.[76] Using the SAG culture collection (Sammlung von Algenkulturen der Universität Göttingen; Culture Collection of Algae at Göttingen University) as the source for various algae, *P. kessleri* was one of four species from various genera able to adapt rapidly to growth in medium containing 10% of the treated waste resulting in high biomass and fatty acid yields. Also, *P. kessleri* was used to reduce glucose content and increase lipids from brewery waste water.[77] The results indicated that *P. kessleri* could have beneficial effects on wastewater from sources in addition to municipal waste water.

<u>Bioremediation of and interaction with other materials</u>
Very short-lived halocarbons are generated by growing marine organisms. Biogenic volatile halocarbon production was observed for a diatom (*Amphora* sp.), a cyanobacteria (*Synechococcus* sp.) and a green alga (*Parachlorella* sp.). The halocarbons may contribute to stratospheric catalytic destruction of ozone. The lowest halocarbon-producing organism studied in this group was *Parachlorella*.[78]

P. kessleri was shown to degrade benzo[a] pyrene in cultures of the algae.[79] Degradation of the chemical was detected using mass spectrometry. In contrast to photooxidation of Benzo[a]pyrene, *P. kessleri* degraded the chemical to CO_2. Live cell mass was considerably more efficient at the degradation than was dead cell mass indicating that the compound was metabolized.

Anabaena oryzae and *Chlorella* (*Parachlorella*) *kessleri* were tested separately and grown together in a range of crude oil concentrations over periods up to 15 days.[80] Crude oil hydrocarbons were the sole carbon source in the cultures. *P. kessleri* growth autotrophically or with *Anabaena* mixotrophically increased in density as measured using chlorophyll light absorbance in the presence of 1% crude oil, although changes in cell mass measured using dry weight or carbohydrate content did not increase as much as the chlorophyll data indicated. The *Anabaena* or *Parachlorella* grown with 1% crude oil were both able to substantially reduce numerous hydrocarbons in autotrophic and mixotrophic cultures over a 30-day period. The results indicate that the organisms may be useful in reducing hydrocarbon contamination in water, although many details of optimal mixotrophic cultures remain to be worked out.

Parachlorella kessleri was tested for biodegradation of benzene, toluene, ethylbenzene and xylenes (BTEX) under model conditions.[81] BTEX-mixture was added to the cultures as the sole carbon source, at a concentration of 100 µg L^{-1}. Loss of BTEX was determined after 24, 48 and 72 h. Benzene and xylenes were degraded by 40 % within 48 h. The highest toluene degradation was 63 %. Only 30 % of ethylbenzene was degraded after 72 h. On the other hand, the elementary analysis of algal biomass was assayed before and after the biodegradation process. After biodegradation the carbon/nitrogen ratio in the biomass was increased 2.7 times.

Impact on Human Health

Effect on human food chain

A recent review described the effect of taxonomic changes on the consequences of the use of *Chlorella* and related species as food in Europe.[82] *Chlorella* has been applied as a nearly generic name for a variety of coccoid green algae used as food for many years. The review describes the inventory of specific strains and species under the name "*Chlorella*" traditionally consumed in Europe. The goal was to provide a list of "*Chlorella*" species that might have been consumed in Europe before 1997 and would therefore not be subject to the Novel Food Regulation rules in Europe. As noted in the introduction to the review: "The coccoid microalgae Chlorella is one of the most important commercial microalgae. The world production was up to 2,000 tons dry weight in 2005 … The main market for Chlorella is human nutrition, the dried powder being processed as powder, capsules or tablet …" The review noted that the "evolution of classification methods has led to modifications of the taxonomy of *Chlorella* … thus it seems that the three species listed in the Novel Food Catalogue – '*Chlorella pyrenoidosa'*, '*Chlorella vulgaris'*, and '*Chlorella luteoviridis'* – might be commercial names rather than true taxa. Moreover, their adequacy with current taxonomy is questioned." Champenois, et al.[82] provided examples of strains assigned to different genera based on recent taxonomic revisions, as shown in the table below. The authors noted that the name "*C. kessleri*" is found, apparently for historical reasons, in some culture collections assigned to strains that have been used for decades in nutritional applications and where the currently valid name is *Parachlorella kessleri* or *P. kessleri*. Additionally, as noted in the table, the species name "*Chlorella vulgaris*" has been used for strains that should now be renamed as *P. kessleri*.

Former Name	**Current Name**	**Strain Number**
Chlorella kessleri	*Parachlorella kessleri*	CCAP 211/11G
Chlorella kessleri	*Parachlorella kessleri*	SAG 8.80
Chlorella vulgaris	*Chlorella kessleri*	ATCC 11468
Chlorella vulgaris	*Chlorella kessleri*	CPCC 266
Chlorella vulgaris	*Chlorella kessleri*	UTEX 262
Chlorella vulgaris	*Chlorella kessleri*	UTEX 263
Chlorella vulgaris	*Chlorella kessleri*	UTEX 397
Chlorella vulgaris	*Chlorella kessleri*	UTEX 398
Chlorella vulgaris	*Chlorella kessleri*	"Strain that could have been commercialized and consumed while it was incorrectly named *C. vulgaris."*
Table derived and quoted from Tables 2- 6 in ref. [82]		

The review cites several publications that provide more details regarding taxonomic classification issues in *Chlorella*-related algae as well as more documentation of how the algae have been used in human food. A major conclusion is that *Parachlorella spp.* have been used as human food for many years, although the older nomenclature persists in some literature and food product labels.

Direct toxic or infectious effect in humans

No publications describing direct toxic or infectious effects in humans were found. Conversely, *Parachlorella* algae appears to have been used as human food for many years.

Direct allergenic effect in humans

No reports were found indicating allergic responses to *Parachlorella* or organisms in its clade, and no evidence of allergic reactions to the closely related genus *Chlorella* were found.

Assessment of Impact of Release

The two fully recognized and one provisionally recognized species in the genus *Parachlorella* have similar biological properties, although the type species, *P. kessleri*, has been directly examined most frequently. No adverse bloom formation has been noted despite nearly worldwide documentation of the presence of the genus, especially in major rivers and fresh water large inland lakes. *Parachlorella* habitat also extends to some harsh environments, in particular high temperature bodies of water. *Parachlorella* has been found as a native inhabitant in wastewater, which is considered another harsh environment, especially with regard to nutrient conditions resulting in competition with many other types of aquatic organisms and stressful conditions of hydrogen ion concentrations, salinity, the presence of heavy metals and of herbicides. *Parachlorella* also has shown a remarkable ability to adapt to a wide variety of conditions starting from its already robust tolerance for stressed biological environments.

Despite its widespread presence in fresh and salt waters and its adaptability to biologically harsh environments, *Parachlorella* has not been associated with bloom activity. Instead, *Parachlorella spp.* appear to be important co-species for water remediation with no obvious tendency to become hazardous when present as a native species or when added to a body of water, such as in wastewater treatment in municipal or industrial settings. Harsh environments have been described where beneficial effects of the presence of *Parachlorella* has been reported, such as crude oil fields and run off from a failed nuclear power plant. These reports provide an unusual degree of likelihood that release into the environment of members of the *Parachlorella* clades are unlikely to prove harmful.

Competition between *Parachlorella* when present with other algae also appear to indicate, first that the *Parachlorella* will survive in the presence of toxic prokaryotic algae and also that the beneficial effects of *Parachlorella* may remain, even if with diminished growth capacity, in physically or biologically stressed environments. The ability of *Parachlorella* to remediate biological contamination by carbon, nitrogen and/or phosphorus-containing nutrients is well-documented, and the ability to scavenge and/or chelate heavy metals is also usually considered beneficial. *Parachlorella* spp. are also tolerant to several herbicides. An exception may occur if the algae concentrate toxic metals or other materials and then serve as a primary or sole food source for a higher organism. The later has not been reported in detail but should, perhaps, be the subject of further investigation.

Overall, the presence of *Parachlorella* has been reported as positive for several aquatic environments where it is found as a native species, and introduction into environments where it may not be native did not result in reports of adverse effects. Additionally, no negative reports of contact with humans were found in the context of adverse health effects, including the absence of adverse allergenic activity. Instead, *Parachlorella* has seen widespread use as human food, albeit often under the previously used name of *Chlorella*, nomenclature that has persisted despite current recognition that the old name is taxonomically and phylogenetically incorrect. In short, release of *Parachlorella* appears likely to be innocuous or even beneficial.

Acknowledgements

Database searches based on predefined criteria were performed by Mr. Thomas Perkins under the direction of the Select Bio Consult project leader. Mr. Perkins has more than 20 years of experience designing and conducting searches using a variety of databases in the scientific and technical literature. Mr. Perkins is employed by Nerac, a company located in Tolland, CT, with over 40 years of experience in database compiling, searching and analysis.

Bibliography

1. Krienitz, L.; Bock, C., Present state of the systematics of planktonic coccoid green algae of inland waters. *Hydrobiologia* **2012,** *698* (1), 295-326.
2. Jiang, L.-L.; Wen, X.-B.; Geng, Y.-H.; Mei, H.; Li, Y.-G., A newly selected lipid-rich microalgae strain and its molecular identification. *Acta Hydrobiological Sinica* **2013,** *37* (4), 606-612.
3. Krienitz, L.; Hegewald, E. H.; Hepperle, D.; Huss, V. A. R.; Rohr, T.; Wolf, M., Phylogenetic relationship of Chlorella and Parachlorella gen. nov. (Chlorophyta, Trebouxiophyceae). *Phycologia* **2004,** *43* (5), 529-542.
4. Bock, C.; Pažoutová, M.; Krienitz, L., Phylogenetic position of Coronastrum ellipsoideum and description of Parachlorella hussii sp. nov. *Biologia* **2011,** *66* (4).
5. Shimura, H.; Itoh, K.; Sugiyama, A.; Ichijo, S.; Ichijo, M.; Furuya, F.; Nakamura, Y.; Kitahara, K.; Kobayashi, K.; Yukawa, Y.; Kobayashi, T., Absorption of radionuclides from the Fukushima nuclear accident by a novel algal strain. *PLoS One* **2012,** *7* (9), e44200.
6. Bock, C.; Pröschold, T.; Krienitz, L., Two new Dictyosphaerium-morphotype lineages of the Chlorellaceae (Trebouxiophyceae): Heynigiagen.nov. and Hindakiagen.nov. *European Journal of Phycology* **2010,** *45* (3), 267-277.
7. Bock, C.; Pröschold, T.; Krienitz, L., Updating the Genus Dictyosphaerium and Description of Mucidosphaerium Gen. Nov. (Trebouxiophyceae) Based on Morphological and Molecular Data. *Phycology* **2011,** *47*, 638-652.
8. Škaloud, P.; Němcová, Y.; Pytela, J.; Bogdanov, N. I.; Bock, C.; Pickinpaugh, S. H., Planktochlorella nurekis gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid green alga carrying significant biotechnological potential. *Fottea* **2014,** *14* (1), 53-62.
9. Song, H.; Wang, Q.; Liu, X.; Hu, Y.; Long, J.; Liu, G.; Hu, Z., Phylogenic Diversity and Taxonomic Problems of the Dictyosphaerium Morphotype within the Parachlorella Clade (Chlorellaceae, Trebouxiophyceae). *J Eukaryot Microbiol* **2018,** *65* (3), 382-391.
10. Turmel, M.; Otis, C.; Lemieux, C., The chloroplast genomes of the green algae Pedinomonas minor, Parachlorella kessleri, and Oocystis solitaria reveal a shared ancestry between the Pedinomonadales and Chlorellales. *Mol Biol Evol* **2009,** *26* (10), 2317-31.
11. Heeg, J. S.; Wolf, M., ITS2 and 18S rDNA sequence-structure phylogeny of Chlorella and allies (Chlorophyta, Trebouxiophyceae, Chlorellaceae). *Plant Gene* **2015,** *4*, 20-28.
12. Takeda, H., Cell Wall Composition and Taxonomy of Symbiotic Chlorella from Paramecium and Acanthocystis. *Phytochemistry* **1995,** *40* (2), 457-459.
13. Nemcova, Y.; Kalina, T., Cell wall development, microfibril and pyrenoid structure in type strains of Chlorella vulgaris, C. kessleri, C. sorokiniana compared with C. luteoviridis (Trebouxiophyceae, Chlorophyta). *Archiv für Hydrobiologie, Supplement* **2000,** *136*, 95-105.
14. Huss, V., A. R. ; Frank, C.; Hartmann, E. C.; Hirmer, M.; Kloboucek, A.; Seidel, B. M.; Wenzeler, P.; Kessler, E., Biochemical Taxonomy and Molecular Phylogeny of the Genus Chlorella Sensu Lato (Chlorophyta). *Journal of Phycology* **1999,** *35*, 587-598.
15. Yamamoto, M.; Kurihara, I.; Kawano, S., Late type of daughter cell wall synthesis in one of the Chlorellaceae, Parachlorella kessleri (Chlorophyta, Trebouxiophyceae). *Planta* **2005,** *221* (6), 766-75.
16. McCarren, J., Parachlorella spp. identified and/or isolated by Synthetic Genomics Inc. in its global bioprospecting expeditions. Buxser, S., Ed. 2019.
17. Dirbaz, M.; Roosta, A., Adsorption, kinetic and thermodynamic studies for the biosorption of cadmium onto microalgae Parachlorella sp. *Journal of Environmental Chemical Engineering* **2018,** *6* (2), 2302-2309.

18. Ngo, H. T. T.; Gerstmann, S.; Frank, H., Toxicity of cadmium to the green algaParachlorella kessleri: Producing Cd-loaded algae for feeding experiments. *Toxicological & Environmental Chemistry* **2009,** *91* (2), 279-288.
19. Bajguz, A., Suppression of Chlorella vulgaris growth by cadmium, lead, and copper stress and its restoration by endogenous brassinolide. *Arch Environ Contam Toxicol* **2011,** *60* (3), 406-16.
20. Kessler, E., Limits of growth of five Chlorella species in the presence of toxic heavy metals. *Algological Studies* **1986,** *42*, 123-128.
21. Exposito, N.; Kumar, V.; Sierra, J.; Schuhmacher, M.; Gimenez Papiol, G., Performance of Raphidocelis subcapitata exposed to heavy metal mixtures. *Sci Total Environ* **2017,** *601-602*, 865-873.
22. Horvatić, J.; Peršić, V.; Pavlić, Ž.; Stjepanović, B.; Has-Schön, E., Toxicity of Metals on the Growth of Raphidocelis subcapitata and Chlorella kessleri using Microplate Bioassays. *Fresenius Environmental Bulletin* **2007,** *16* (7), 826-831.
23. Kadukova, J., Surface sorption and nanoparticle production as a silver detoxification mechanism of the freshwater alga Parachlorella kessleri. *Bioresour Technol* **2016,** *216*, 406-13.
24. Schiariti, A.; Juárez, Á. B.; Rodríguez, M. C., Effects of sublethal concentrations of copper on three strains of green microalgae under autotrophic and mixotrophic culture conditions. *Algological Studies* **2004,** *114* (1), 143-157.
25. Nudelman, M. A.; Carro, C.; Nudelman, N. S., Effects of tin(IV) chloride and of organotin compounds on aquatic micro-organisms. *Applied Organometallic Chemistry* **1998,** *12* (1), 67-75.
26. Juárez, Á. B.; Vélez, C. G.; Iñiguez, A. R.; Martínez, D. E.; Rodríguez, M. C.; Vigna, M. S.; de Molina, M. d. C. R., A Parachlorella kessleri (Trebouxiophyceae, Chlorophyta) strain from an extremely acidic geothermal pond in Argentina. *Phycologia* **2011,** *50* (4), 413-421.
27. de Morais, M. G.; Costa, J. A. V., Isolation and selection of microalgae from coal fired thermoelectric power plant for biofixation of carbon dioxide. *Energy Conversion and Management* **2007,** *48* (7), 2169-2173.
28. Vinayakumar, M.; Kessler, E., Physiological and biochemical contributions to the taxonomy of the genus Chlorella X. Products of Glucose Fermentation. *Archives of Microbiology* **1975,** *103* (1), 13-19.
29. Horvatić, J.; Lukavsky, J., Algal growth potential (AGP) and toxicity of the waters of the Stara Drava channels and the Sakadaš Lake (Kopački rit, Croatia). *Algological Studies* **1997,** *86*, 163-170.
30. Horvatić, J.; Palijan, G.; Lukavský, J., Algal responses to nutrient additions in water of Nature Park Kopački rit (Croatia) by Miniaturized Algal Growth Bioassay. *Algological Studies* **2003,** *110* (1), 117-126.
31. Horvatić, J.; Mihaljević, M.; Stević, F., Algal growth potential of Chlorella kessleri FOTT et NOV. in comparison with in situ microphytoplankton dynamics in the water of the marshes of Lake Sakadaš. *Periodicum Biologorum* **2003,** *105* (3), 307-312.
32. Horvatić, J.; Peršić, V.; Mihaljević, M., Bioassay Method in Evaluation of Trophic Conditions and Nutrient Limitation in the Danube Wetland Waters (1388–1426 r. km). *Hydrobiologia* **2006,** *563* (1), 453-463.
33. Peršić, V.; Horvatić, J.; Mihaljević, M., Bioassay method in the trophic evaluation of a wetland area-case study in the Danubian region (1426-1388 r. km). *Periodicum Biologorum* **2005,** *107* (3), 293-298.
34. Peršić, V.; Horvatić, J., Spatial Distribution of Nutrient Limitation in the Danube River Floodplain in Relation to Hydrological Connectivity. *Wetlands* **2011,** *31* (5), 933-944.
35. Horvatić, J.; Čačić, L.; Jurišić, M., Algal growth potential (AGP) of Chlorella kessleri Fott & Nov. in the Drava River. *River Systems* **2003,** *14* (3-4), 267-281.
36. Peršić, V.; Horvatić, J.; Has-Schön, E.; Bogut, I., Changes in N and P limitation induced by water level fluctuations in Nature Park Kopački Rit (Croatia): nutrient enrichment bioassay. *Aquatic Ecology* **2009,** *43* (1), 27-36.

37. El-Sheek, M. M.; Rady, A. A., Effect of phosphorus starvation on growth, photosynthesis and some metabolic processes in the unicellular green alga Chlorella kessleri. *Phyton* **1995,** *35* (1), 139-151.
38. Hammouda, O.; Gaber, A.; Abdelraouf, N., Microalgae and Wastewater Treatment. *Ecotoxicology and Environmental Safety* **1995** *31* (3), 205-210.
39. Abinandan, S.; Shanthakumar, S., Challenges and opportunities in application of microalgae (Chlorophyta) for wastewater treatment: A review. *Renewable and Sustainable Energy Reviews* **2015,** *52*, 123-132.
40. Whitton, R.; Ometto, F.; Pidou, M.; Jarvis, P.; Villa, R.; Jefferson, B., Microalgae for municipal wastewater nutrient remediation: mechanisms, reactors and outlook for tertiary treatment. *Environmental Technology Reviews* **2015,** *4* (1), 133-148.
41. Cho, D. H.; Choi, J. W.; Kang, Z.; Kim, B. H.; Oh, H. M.; Kim, H. S.; Ramanan, R., Microalgal diversity fosters stable biomass productivity in open ponds treating wastewater. *Sci Rep* **2017,** *7* (1), 1979.
42. Osundeko, O.; Dean, A. P.; Davies, H.; Pittman, J. K., Acclimation of microalgae to wastewater environments involves increased oxidative stress tolerance activity. *Plant Cell Physiol* **2014,** *55* (10), 1848-57.
43. Arbib, Z.; Ruiz, J.; Alvarez-Diaz, P.; Garrido-Perez, C.; Perales, J. A., Capability of different microalgae species for phytoremediation processes: wastewater tertiary treatment, CO2 bio-fixation and low cost biofuels production. *Water Res* **2014,** *49*, 465-74.
44. Osundeko, O.; Davies, H.; Pittman, J. K., Oxidative stress-tolerant microalgae strains are highly efficient for biofuel feedstock production on wastewater. *Biomass and Bioenergy* **2013,** *56*, 284-294.
45. Zeraatkar, A. K.; Ahmadzadeh, H.; Talebi, A. F.; Moheimani, N. R.; McHenry, M. P., Potential use of algae for heavy metal bioremediation, a critical review. *J Environ Manage* **2016,** *181*, 817-831.
46. Bulgariu, L.; Gavrilescu, M., Bioremediation of Heavy Metals by Microalgae. In *Handbook of Marine Microalgae*, 2015; pp 457-469.
47. Suresh Kumar, K.; Dahms, H. U.; Won, E. J.; Lee, J. S.; Shin, K. H., Microalgae - A promising tool for heavy metal remediation. *Ecotoxicol Environ Saf* **2015,** *113*, 329-52.
48. Rojíčková, R.; Maršálek, B., Selection and sensitivity comparisons of algal species for toxicity testing. *Chemosphere* **1999,** *38* (14), 3329-3338.
49. Ibuot, A.; Dean, A. P.; McIntosh, O. A.; Pittman, J. K., Metal bioremediation by CrMTP4 over-expressing Chlamydomonas reinhardtii in comparison to natural wastewater-tolerant microalgae strains. *Algal Research* **2017,** *24* (A), 89-96.
50. Spoljaric Maronic, D.; Stolfa Camagajevac, I.; Horvatic, J.; Zuna Pfeiffer, T.; Stevic, F.; Zarkovic, N.; Waeg, G.; Jaganjac, M., S-metolachlor promotes oxidative stress in green microalga Parachlorella kessleri - A potential environmental and health risk for higher organisms. *Sci Total Environ* **2018,** *637-638*, 41-49.
51. Mostafa, F. I. Y.; Helling, C. S., Isoproturon degradation as affected by the growth of two algal species at different concentrations and pH values. *Journal of Environmental Science and Health, Part B* **2001,** *36* (6), 709-727.
52. Deng, D.; Tam, N. F., Isolation of microalgae tolerant to polybrominated diphenyl ethers (PBDEs) from wastewater treatment plants and their removal ability. *Bioresour Technol* **2015,** *177*, 289-97.
53. Pereira, A. L.; Santos, C.; Azevedo, J.; Martins, T. P.; Castelo-Branco, R.; Ramos, V.; Vasconcelos, V.; Campos, A., Effects of two toxic cyanobacterial crude extracts containing microcystin-LR and cylindrospermopsin on the growth and photosynthetic capacity of the microalga Parachlorella kessleri. *Algal Research* **2018,** *34*, 198-208.
54. Park, S.; Van Ginkel, S. W.; Pradeep, P.; Igou, T.; Yi, C.; Snell, T.; Chen, Y., The Selective Use of Hypochlorite to Prevent Pond Crashes for Algae-Biofuel Production. *Water Environ Res* **2016,** *88* (1), 70-8.

55. Peng, C.-c.; Li, Z.-j.; Cao, Y.-c.; Liu, X.-z.; Hu, X.-j., Change of dominant species of planktonic microalgae in Litopenaeus vannamei's semi-intensive culture ponds and its impact on the culture environment. *Marine Environmental Science* **2011,** *30* (2), 193-198.
56. Ueno, M.; Itami, T.; Yamashita, K.; Oda, T., Beneficial effects of dietary supplemented Parachlorella kessleri strain KNK-A001 on pacific white shrimp (Litopenaeus vannamei) and kuruma shrimp (Marsupenaeus japonicus). *Aquaculture Science* **2016,** *64* (2), 201-204.
57. Chaufan, G.; Juarez, A.; Basack, S.; Ithuralde, E.; Sabatini, S. E.; Genovese, G.; Oneto, M. L.; Kesten, E.; Rios de Molina Mdel, C., Toxicity of hexachlorobenzene and its transference from microalgae (Chlorella kessleri) to crabs (Chasmagnathus granulatus). *Toxicology* **2006,** *227* (3), 262-70.
58. Doicheva, L.; Fournadzhieva, S.; Pilarski, P.; Andreeva, R.; Nyagulov, L., Investigation of the Complex Influence of the Preparation Stomp E33 (Pendimethalin) Over Daphnia Magna Straws (Dissolved in Water and Accumulated from Chlorella Kessleri). *Biotechnology & Biotechnological Equipment* **2009,** *23* (1), 301-304.
59. Pradeep, V.; Van Ginkel, S. W.; Park, S.; Igou, T.; Yi, C.; Fu, H.; Johnston, R.; Snell, T.; Chen, Y., Use of Copper to Selectively Inhibit Brachionus calyciflorus (Predator) Growth in Chlorella kessleri (Prey) Mass Cultures for Algae Biodiesel Production. *Int J Mol Sci* **2015,** *16* (9), 20674-84.
60. Swackhamer, D. L.; Skoglund, R. S., The role of phytoplankton in the partitioning of hydrophobic organic contaminants in water. In *Organic Substances and Sediments in Water: Processes and Analytical*, Baker, R. A., Ed. Lewis Publishers: 1991; Vol. 2, pp 91-105.
61. Lederman, T. C.; Rhee, G.-Y., Bioconcentration of a Hexachlorobiphenyl in Great Lakes Planktonic Algae. *Canadian Journal of Fisheries and Aquatic Sciences* **1982,** *39* (3), 380-387.
62. Schneider, S. C.; Nizzetto, L., Bioconcentration and intracellular storage of hexachlorobenzene in charophytes and their potential role in monitoring and remediation actions. *Environ Sci Technol* **2012,** *46* (22), 12427-34.
63. Kessler, E.; Huss, V. A. R.; Rahat, M., Species-specific ability of Chlorella strains (Chlorophyceae) to form stable symbioses with Hydra viridis. *Plant Systematics and Evolution* **1998,** *160* (3-4), 241-246.
64. Noguchi, N.; Konishi, F.; Kumamoto, S.; Maruyama, I.; Ando, Y.; Yanagita, T., Beneficial effects of Chlorella on glucose and lipid metabolism in obese rodents on a high-fat diet. *Obes Res Clin Pract* **2013,** *7* (2), e95-e105.
65. Ishiguro, S.; Uppalapati, D.; Goldsmith, Z.; Robertson, D.; Hodge, J.; Holt, H.; Nakashima, A.; Turner, K.; Tamura, M., Exopolysaccharides extracted from Parachlorella kessleri inhibit colon carcinoma growth in mice via stimulation of host antitumor immune responses. *PLoS One* **2017,** *12* (4), e0175064.
66. Ueno, M.; Cho, K.; Hirata, N.; Yamashita, K.; Yamaguchi, K.; Kim, D.; Oda, T., Macrophage-stimulating activities of newly isolated complex polysaccharides from Parachlorella kessleri strain KNK-A001. *Int J Biol Macromol* **2017,** *104* (Pt A), 400-406.
67. Uchikawa, T.; Kumamoto, Y.; Maruyama, I.; Kumamoto, S.; Ando, Y.; Yasutake, A., The enhanced elimination of tissue methylmercury in Parachlorella beijerinckii-fed mice. *Journal of Toxicological Sciences* **2011,** *36* (1), 121-126.
68. Uchikawa, T.; Ueno, T.; Hasegawa, T.; Maruyama, I.; Kumamoto, S.; Ando, Y., Parachlorella beyerinckii accelerates lead excretion in mice. *Toxicol Ind Health* **2009,** *25* (8), 551-6.
69. Li, Y.; Zhou, W.; Hu, B.; Min, M.; Chen, P.; Ruan, R. R., Effect of light intensity on algal biomass accumulation and biodiesel production for mixotrophic strains Chlorella kessleri and Chlorella protothecoide cultivated in highly concentrated municipal wastewater. *Biotechnol Bioeng* **2012,** *109* (9), 2222-9.
70. Osundeko, O.; Pittman, J. K., Implications of sludge liquor addition for wastewater-based open pond cultivation of microalgae for biofuel generation and pollutant remediation. *Bioresour Technol* **2014,** *152*, 355-63.

71. Lv, J.; Wang, X.; Feng, J.; Liu, Q.; Nan, F.; Jiao, X.; Xie, S., Comparison of growth characteristics and nitrogen removal capacity of five species of green algae. *Journal of Applied Phycology* **2018,** *31* (1), 409-421.
72. Álvarez-Díaz, P. D.; Ruiz, J.; Arbib, Z.; Barragán, J.; Garrido-Pérez, M. C.; Perales, J. A., Freshwater microalgae selection for simultaneous wastewater nutrient removal and lipid production. *Algal Research* **2017,** *24*, 477-485.
73. Kumar Singh, A.; Sharma, N.; Farooqi, H.; Zainul Abdin, M.; Mock, T.; Kumar, S., Phycoremediation of municipal wastewater by microalgae to produce biofuel. *International Journal of Phytoremediation* **2017,** *19* (9), 805-812.
74. Zhang, Y.; Ma, N.; Fu, T.; Meng, J.; Zhou, W., Treatment of Nutrient-rich Municipal Wastewater Using Mixotrophic Strain Chlorella kessleri GXLB-9. *Journal of Sustainable Development Studies* **2014,** *7* (1), 1-18.
75. Mahdavi, H.; Ulrich, A. C.; Liu, Y., Metal removal from oil sands tailings pond water by indigenous micro-alga. *Chemosphere* **2012,** *89* (3), 350-4.
76. Koutra, E.; Grammatikopoulos, G.; Kornaros, M., Selection of microalgae intended for valorization of digestate from agro-waste mixtures. *Waste Manag* **2018,** *73*, 123-129.
77. O'Rourke, R.; Gaffney, M.; Murphy, R., The effects of Parachlorella kessleri cultivation on brewery wastewater. *Water Sci Technol* **2016,** *73* (6), 1401-8.
78. Lim, Y.-K.; Phang, S.-M.; Sturges, W. T.; Malin, G.; Rahman, N. B. A., Emission of short-lived halocarbons by three common tropical marine microalgae during batch culture. *Journal of Applied Phycology* **2018,** *30* (1), 341-353.
79. Takáčová, A.; Smolinská, M.; Ryba, J.; Mackuľak, T.; Jokrllová, J.; Hronec, P.; Čík, G., Biodegradation of Benzo[a]Pyrene through the use of algae. *Central European Journal of Chemistry* **2014,** *12* (11), 1133-1143.
80. Hamouda, R. A. E. F.; Sorour, N. M.; Yeheia, D. S., Biodegradation of crude oil by Anabaena oryzae , Chlorella kessleri and its consortium under mixotrophic conditions. *International Biodeterioration & Biodegradation* **2016,** *112*, 128-134.
81. Takáčová, A.; Smolinská, M.; Semerád, M.; Matúš, P., Degradation of BTEX by Microalgae Parachlorella kessleri. *Petroleum & Coal* **2015,** *57* (2), 101-107.
82. Champenois, J.; Marfaing, H.; Pierre, R., Review of the taxonomic revision of Chlorella and consequences for its food uses in Europe. *Journal of Applied Phycology* **2015,** *27* (5), 1845-1851.

Appendices

Databases Searched

BIOSIS, Medline, Scopus and Proquest databases were searched on September 4, 2018. Searches covered titles and abstracts. Search results were compiled from the various sources and replicates were removed. A total of 751 unique citations were obtained.

Search Strategies

Broad search terms were used to select as many citations as practical. The search terms were "P. beijerinckii" or "Parachlorella beijerinckii" or "C. beijerinckii" or "chlorella beijerinckii" or "P. kessleri" or "Parachlorella kessleri" OR "P. hussii" or "Parachlorella hussii" or "Parachlorella". Each title and abstract were examined for relevant content and 213 relevant citations were selected for close examination in full text form. Further refinement of the search terms was not necessary since fewer than 1000 citations identified mentioned species names.

Papers identified by search but not cited in the body of the report

Literature reviewed but not cited is included in a spreadsheet accompanying the report. The spreadsheet contains abstracts for the articles not cited as well as for the articles cited above.

Details of sites where *Parachlorella* spp. have been isolated

Species	**Location**
P. kessleri	Lawn soil, Shanxi Province, China
P. hussii	Secondary waste treatment water, Cheshire, United Kingdom
C. kessleri	River, Nanning, China
P. beijerinckii	Brook, West Pomerania, Germany
P. kessleri	Soil, Shanxi Province, China
P. kessleri	Bay of Bengal, India
P. kessleri	Isothermal acidic pond, Leguna Verde, Neuquen, Argentina
P. kessleri	Activated sludge wastewater treatment pond, Ibaraki, Japan
P. sp.	Southern Iran
P. kessleri	Bay of Bengal, India
P. kessleri	Drava River, Croatia
P. kessleri	Kopacˇki Rit, eastern Croatia (confluence of the Danube and Drava rivers), Sakadas, Croatia
P. kessleri	Freshwater, New York City, New York
P. kessleri	River water, Tanta City, Egypt
P. kessleri	Laguna Verde, Neuquen, Argentina
P. kessleri	Armenia
P. kessleri	Indian Ocean
P. kessleri	River water, Hanover, Germany
P. kessleri	Thermal pond, Neuquen, Argentina
P. kessleri	Buenos Aires, Argentina
P. kessleri	Fish pond, University of Pennsylvania, Philadelphia, Pennsylvania
Parachlorella sp.	East coast waters, Teremgganu, Malaysia
P. kessleri	Subaerial sites, Murcia region, Spain
P. kessleri	Ukraine
P. kessleri	Rice paddy fields, Egypt

Species	Location
P. kessleri	Botanic garden Cienfuegos, Cuba
P. kessleri	Harbor, New York
P. kessleri	Public fountains, Bratislava, Slovakia
P. hussii	Wastewater, Ellesmere Port, Cheshire, UK
Parachlorella sp. (kessleri)	Republic of Korea
P. kessleri	Thermoelectric plant, Rio Grande do Sul, Brazil
P. kessleri	Dammam, Saudi Arabia
Parachlorella sp. (related to kessleri)	Wastewater plants, Hong Kong, China
Parachlorella sp.	Sea bass pond, Sepang, Selangor, Malaysia (8 strains)
Parachlorella sp.	Seawater, Terengganu, Malaysia
Parachlorella sp.	Farm, University of Malaya, Kuala Lumpur, Malaysia
P. kessleri	Water from bitumen extraction facility, Fort McMurray, Alberta, Canada
P. hussii	Vala de Maiorca, Portugal
P. hussii	Rock pool, Serra do Geres, Rio Hemem (Homem River), Portugal
P. hussii	Pond, Serra do Geres, Rio Homem (Homem River), Portugal
P. hussii	Plant squeezing, lake north of Lagoa da Murta, Brazil
P. hussii	Pond, New York State
Parachlorella morphotype	Lake Victoria, Kenya
P. kessleri	Outdoor culture unit, Trebon, Czech Republic
Parachlorella sp.	SGI: San Diego County; Imperial County (Calipatria); Salton Sea, California
Parachlorella sp.	SGI: Hillsborough County (aquaculture pond); Lee and Collier County (Gulf Coast), Florida
Parachlorella sp.	SGI: Kaneohe Bay; Mamala Bay, O'ahu; Hilo Bay, Hawaii;
Parachlorella sp.	SGI: Great Salt Lake (ephemeral pool), Utah
Parachlorella sp.	SGI: South Padre Island; Trinity Bay; Jefferson County (Gulf Coast); Lake Texana; Calhoun County (aquaculture pond), Texas
Parachlorella sp.	SGI: Multiple inland ponds; NW, W, SW coastlines, Puerto Rico
Parachlorella sp.	SGI: Gulf Coast Mangrove forest; Gulf Coast Marsh, Sinaloa, Mexico
Parachlorella sp.	SGI: Cozumel and Cancun (Gulf Coast), Quintana Roo, Mexico

Author Biography with weblinks

Stephen Buxser, Ph.D. (www.selectbioconsult.com) has more than 30 years of experience in microbiology, cell biology, and biochemistry. His education in microbiology included both medical and environmental topics. With degrees in microbiology, Dr. Buxser has applied his education to areas in the pharmaceutical industry and in consulting with multiple companies in the pharmaceutical, toxicological, environmental, and medical and biotechnology fields. More than 20 years of experience working in and supervising labs in the pharmaceutical industry provided substantial direct hands-on experience in biological sciences and biochemistry and also in immunology, enzyme analysis and kinetics, and biostatistics. From 2003 to 2008, Dr. Buxser was a co-founder of a contract and basic research company focusing on new compounds useful for antimicrobial applications. In addition to research and science supervisory experience, he implemented new intellectual property programs. Consulting experience includes analysis of patents and intellectual property. Statistical expertise includes extensive experience in design and analysis of bioassays at levels extending from biochemical/enzymological, to prokaryotic and eukaryotic whole cells, to whole animal studies encompassing toxicology, pharmacology, immunology, and biostatistics. Over the last 10 years, Dr. Buxser has worked with a variety of companies requiring evaluation of environmental impact of chemicals and organisms, especially microorganisms. This includes more than six years directly engaged in analysis and evaluation of algae in the environment. More than 10 years of consulting experience provided more breadth of experience into areas including medical and environmental microbiology. Dr. Buxser has published more than 50 peer-reviewed articles.

Credentials
Post-Doctoral Fellow, Department of Biochemistry, University of Massachusetts Medical School
Ph.D., Microbiology, University of Cincinnati School of Medicine
M.S., Bacteriology, University of Wisconsin, Madison
B.S. Microbiology, Ohio State University, Columbus, Ohio